Action of *Trypanosoma rangeli* in Infections with Virulent *Trypanosoma cruzi* Populations

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In experimental murine infections with Trypanosoma rangeli *it has been observed development immune response to* Trypanosoma cruzi. *The aim of the present work was to analyze the result of antigenic stimuli and the protective effect with* T. rangeli *in* T. cruzi *infections. Mice groups immunized with metacyclic trypomastigotes of* T. rangeli *(Choachí-2V strain), derived from haemolymph and salivary gland and reinfected with* T. cruzi *virulent populations (Tulahuen strain, SA strain and Dm28c clone) from infected in vitro cells, showed decrease severity of disease outcomes, low parasitemia levels and 100% survival of all mice immunized, in comparison with groups infected only with* T. cruzi *populations, which demonstrated tissue affection, high parasitemia levels and the death of all animals. The above mentioned data contribute to understand the biological behaviour of* T. cruzi *and* T. rangeli *and their interaction with vertebrate host.*

Key words*: Trypanosoma cruzi - Trypanosoma rangeli - protective effect - Chagas disease - parasitemia level*

American trypanosomiasis (Chagas disease), caused by the intracellular protozoan *Trypanosoma cruzi*, is a complicated and devastating disease that affects around 20 million people in Latin America (Miles 1983, Moncayo 1992). Classical transmission to human is by haematophagus triatomine bugs but also by blood transfusions, congenital transplacental, organ transplantation and laboratory accidental infections (Nickerson et al. 1989). Under natural conditions *T. cruzi* infects in the American continent over 100 mammalian species from different orders, including domestic and sylvatic animals (Teixeira et al. 2001). *T. rangeli*, an assumed harmless human parasite, uses also a wide variety of mammals host and hemipterous insects in its life cycle (Urdaneta-Morales & Tejero 1992). It has been postulated that immuno modulating mechanisms induced by *T. rangeli* may have relevance to protection against *T. cruzi* infection (Saldaña & Sousa 1996, Zúñiga et al. 1997a).

Murine models are powerful tools for the study of cellular and humoral immunity to *T. cruzi* and to understand immunological disturbances caused by the infection. Inbred strains of mice may vary from highly resistant to highly susceptible, suggesting a genetic basis for this natural resistance (Hoft et al. 1993) but in neither, the hu-

man infection nor the murine model the factors that contribute to resistance or susceptibility have been completely elucidated.

In the present report we have compared the evolution of the *T. cruzi* infection in Balb/c mice inoculated with 10 000 trypomastigotes from the Chilean Tulahuen strain, the Colombian SA strain or the Venezuelan clone Dm 28c of the parasite, including a protection study by using preinfection with the Colombian Choachí-2V strain of *T. rangeli*.

MATERIALS AND METHODS

Mice stocks - Three and 5 weeks old males from Balb/c inbred mouse strain (bred in the colony of Instituto Nacional de Salud, Bogotá, Colombia) were used for experiments.

All animal studies were conducted in accordance with principles and procedures outlined in the International Guiding Principles for Biomedical Research involving Animals (Council for International Organizations of Medical Sciences).

Parasites stocks - The Tulahuen (Pizzi 1957), SA strains, Dm 28c clone (Contreras et al. 1988) of *T. cruzi* and Choachí-2V strain of *T. rangeli* were used. The SA strain was isolated in 1999, from *Rhodnius pallescenses*, in the San Antonio region, Magdalena, Colombia, where an outbreak of acute Chagas disease occurred that year, with several fatal human cases. The Choachí-2V strain was purified by isolation of parasites from haemolymph and salivary glands of *R. prolixus* experimentally infected with parasites from axenic culture.

Cellular infection - Metacyclic parasites from axenic culture of each *T. cruzi* population, were poured over a semiconfluent layer of Vero cells in Dulbecco's Modified Eagle's Medium (DME) and incubated at 37°C overnight. The medium was then discarded, and fresh DME was added.

Ten days after infection, culture medium was collected and the concentration of live metacyclic trypomastigotes determined by using a hemocytometer.

Vector infection - Thirty fifth instar nymphs of *R. prolixus* maintained in a colony were inoculated by intracelomic route with 2 µl of exponential growth phase axenic culture of *T. rangeli* Choachí-2V strain. Then the triatomines were fed on normal mice and maintained under controlled temperature and humidity conditions. Nine days after infection, metatrypomastigotes were collected from haemolymph and salivary glands and then used to infect Balb/c mice.

Infection model - Five weeks-old mice were always used as hosts to *T. cruzi* infection. Groups of 10 mice (5 weeks old), were intraperitoneally infected with 10 000 metacyclic trypomastigotes/mouse obtained from Vero cells infected with Tulahuen strain, SA strains or Dm 28c clone of *T. cruzi*. In addition, a group of 30 mice (3 weeks old) was infected with 10 000 trypomastigotes/mouse of *T. rangeli* Choachí-2V strain trypomastigotes obtained from haemolymph and salivary glands of *R. prolixus*. Two weeks later, when *T. rangeli* parasitemia was negative under microscope observation, separated groups of mice were reinfected with each 1 of the 3 populations of *T. cruzi*.

Mice from each group were sacrificed at different times after infection and their heart and skeletal muscle were subjected to histopathological study to determine presence of parasite pseudocysts and/or tissue damage in 5 µm tissue sections examined by conventional staining with haematoxylyn and eosin. A group of mice, which received only phosphate buffer saline was used as a control.

Parasitemia measurements - Every 2 days, parasitemia levels were analyzed until mice die or they were negative. To determine the presence and amount of parasites, blood samples from each mouse were collected in heparinized microhematocrit capillaries, from caudal vein. After centrifugation and calculating the blood and plasma volumes, capillaries were cut at the division zone between the buffy coat and the red blood cell sediment. The free-swimming bloodstream trypomastigotes, under a 22 x 22 mm coverslip, were counted in 50 fields at X 400 magnification (Arias & Ferro 1988) .

RESULTS

Mice from Balb/c strain were extremely susceptible to the infection with 10 000 metacyclic trypomastigotes from Tulahuen, SA strains and Dm28c clone of *T. cruzi*. All mice died within 14 days post-infection (p.i.) when infected with Tulahuen strain, within 12 days p.i. with Dm28c clone and within 18 days p.i. when infected with SA strain of *T. cruzi*. The mice infected with Choachí-2V strain, from haemolymph and salivary glands of *R. prolixus*, showed 100% survival after several months p.i., but they had significantly lower parasitemia level than the animals infected with *T. cruzi* parasites. Mice infected with Choachí-2V strain from axenic culture showed scarcity of parasites in circulation (about 10^4 parasites/ml). Fig. 1 shows the evolution of the parasitemia in mice infected with the different parasite populations. The animals infected with Tulahuen and Dm28c clone showed similar

maximun parasitemia peak levels of 2.6 x 10^5 parasites/ml (7 days p.i.) and 2.4 x 10^5 parasites/ml (9 days p.i.), respectively.

In the mice infected with SA strain maximun parasitemia level found to be only 3.2 x 10^4 parasites/ml (10 days p.i.), but the general state of the mice was bad, with presence of parasites in ascitic fluid. The maximun parasitemia level in mice infected with *T. rangeli* strain was of 1.8 x 10^4 parasites/ml.

The mice previously infected with trypomastigotes of *T. rangeli* Choachí-2V strain and later on reinfected with the different *T. cruzi* populations showed 100% survival after 6 weeks of reinfection. The levels of parasitemia were very low (10^3) compared with the levels found in the mice infected only with the *T. cruzi* populations (Fig. 2).

The effect of parasite infection on tissue pathology was evaluated by comparing histopathological study of different tissues obtained at days 5, 10, 12, 20 and 30 p.i. In the first 5 days of infection no significant differences in tissue damage were observed when mice infected with *T. rangeli* were compared with those infected with *T. cruzi*. However, the histopathological study in mice infected with Tulahuen strain and Dm28c clone demonstrated cardiomy-
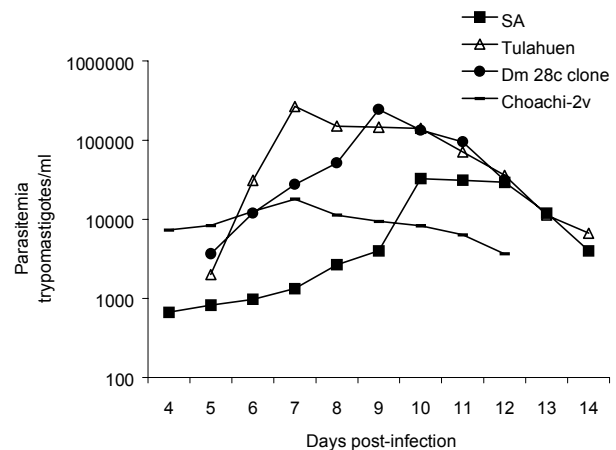

Fig. 1: parasitemia levels expressed as parasites/ml in infected mice with *Trypanosoma cruzi* populations and *Trypanosoma rangeli* strain. Each point represents the mean parasitemia level in the blood of mice groups (n = 10). The graphic is in logarithmic scale.
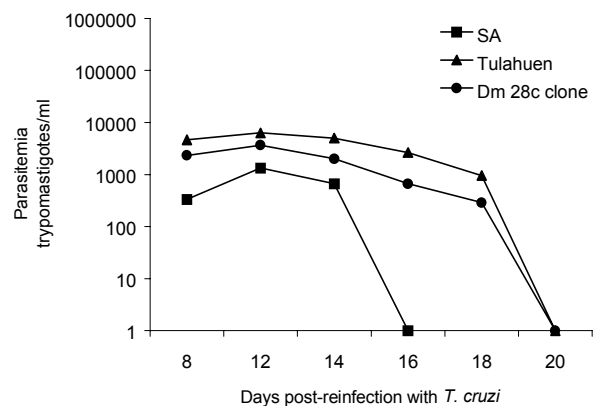

Fig. 2: parasitemia levels in mice group that were infected with *Trypanosoma rangeli* and reinfected at 15 days with *Trypanosoma cruzi* populations. The graphic is in logarithmic scale.

opathy, acute myocarditis and myositis after 10 days p.i. In addition, multiple pseudocysts in cardiac fibers (Fig. 3a) and skeletal muscle were found. As far as the SA strain, we found parasites in intestinal crypts, lym-
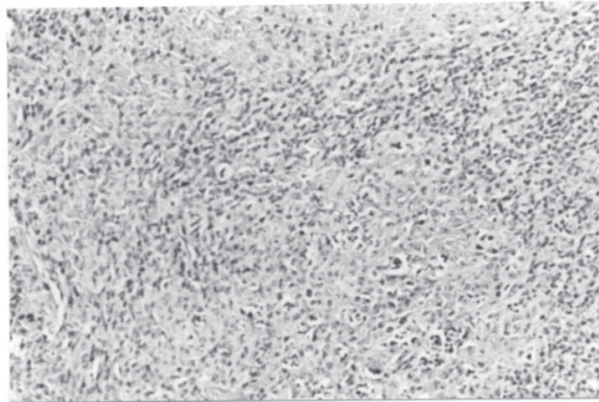
phoplasmocytic inflammation in skeletal muscles, and amastigote nests and tissue damage in ventricular cardiac fibers (Figs 4a, b, c). In the reinfected groups, we found moderate focal myocarditis without amastigote
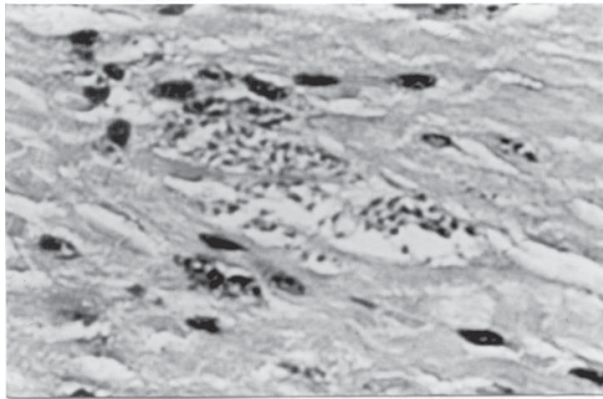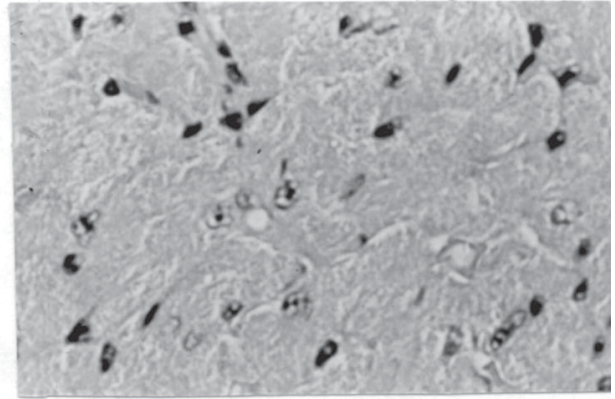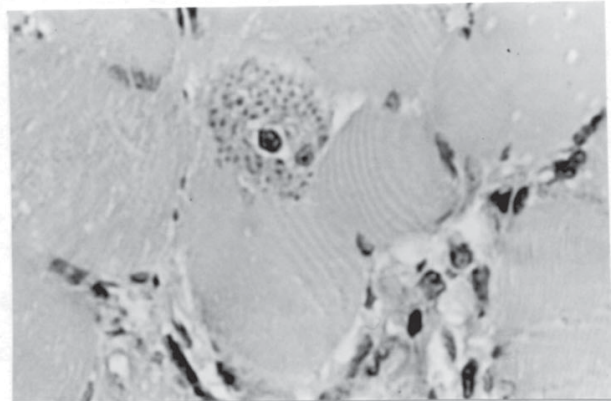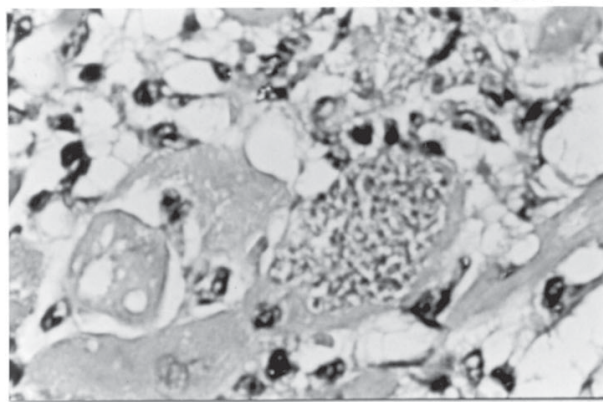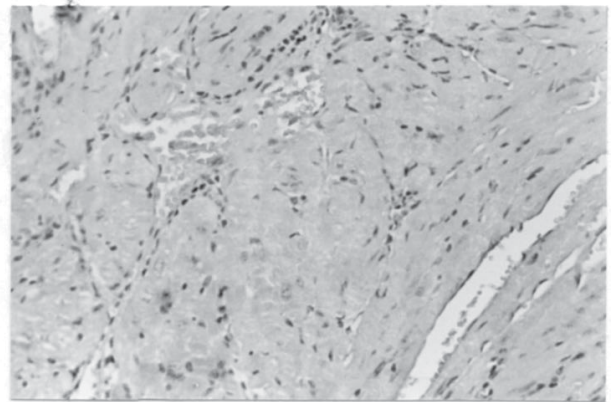

Fig. 3a: Tulahuen strain pseudocysts surrounded by leucocyte infiltration with PMN neutrophils, lymphocytes and macrophages. Myocardial tissue section from infected mouse, 12 days p.i. X 400. Fig 3b: mild cellular infiltration among muscle fibers. Myocardial tissue section from Balb/c mouse infected with *Trypanosoma rangeli* and 15 days later reinfected with Tulahuen strain of *Trypanosoma cruzi*. Myocardial tissue section from infected mouse, 12 days p.i. X 400. Fig. 4a: infiltration and cellular infiltrate among muscle fibers. Myocardial tissue section from Balb/c mouse with SA strain of *T. cruzi*, 10 days p.i. X 100. Fig. 4b: mild cellular infiltration among muscle fibers from Balb/c mouse infected with the SA strain of *T. cruzi*. A pseudocyst is observed in myocardial tissue section from infected mouse, 10 days p.i. X 400. Fig. 4c: detail showing intense acute inflammatory process with PMN neutrophils, lymphocytes and macrophages infiltrate. Disruption of myofibrils and necrosis is observed. Myocardial tissue section of SA infected mouse, 12 days p.i. X 400. Fig. 4d: cellular infiltration among muscle fibers. Myocardial tissue section from Balb/c mouse infected with *T. rangeli* and 15 days later reinfected with the SA strain of *T. cruzi*. Myocardial tissue section from infected mouse, 12 days post-reinfection. X 100.

nests (Figs 3b, 4d), lymphohistiocytic inflammation in skeletal muscles without parasite presence, and normal liver and spleen. On the other hand, the histopathological study of *T. rangeli* infected mice showed no sign of tissue damage. Failure to detect *T. rangeli* amastigotes in the 5 μm tissue sections of the experimentally infected mice could be attributed to the scarcity of intracellular forms and to the possibility that parasites could be localized in lymphoreticular tissues such as bone marrow and lymph nodes, which were not examined in this study.

In summary, mice infected with *T. cruzi* showed fatal chagasic heart disease with increased myocarditis, severe diffuse lesions with mononuclear infiltration, high number of pseudocysts and 100% mortality. The cellular components of inflammatory infiltrate were predominantly macrophages and lymphocytes with focal infiltration of polymorphonuclear neutrophils (Fig. 4c).

DISCUSSION

The study of Chagas disease in animal models allowed the definition of a number of distinct mechanisms of pathogenesis, including parasite antigen-specific inflammation, parasite-induced myocardial cell necrosis and repair and autoimmunity (Leon & Engman 2001).

There is a wide variety in the outcome of *T. cruzi* infection in mouse models. A mouse strain may develop many different types of disease depending on the virulence of the parasite isolate and a single parasite clone may have very different behaviour in resistant and susceptible mouse strains (Leon & Engman 2001).

The Tulahuen and SA strains and Dm28c clone of *T. cruzi* studied in this work, have been analyzed in mice groups, with a remarkable maintenance of morphological characteristic of the host-parasite relationship. *T. cruzi* populations have a clonal structure considered as constitutive units of evolution, which may play a key role in the outcome of Chagas disease (Tibayrenc et al. 1986, Tibayrenc & Ayala 1991, Montilla et al. 2002). Although our parasite strains may be formed by diverse clones (Dvorak 1984), they probably have a permanent and major clonal composition with stable characteristic.

Relationship between parasitemia and mortality of the mice infected here, showed different patterns for Tulahuen strain and Dm28c clone compared with SA strain of *T. cruzi*. In infections with the Tulahuen strain and Dm28c clone, peak of the parasitemia occurred on the 7 and 9 days p.i. with high number of circulating parasites and all mice died within 12 to 14 days of infection. In contrast, all mice infected with SA strain died around 18-22 days p.i., but they showed low parasitemia level, suggesting that the disease is not a direct result of the parasite amount (Bestetti 1997). This high virulence is uncommon in Colombian strains of *T. cruzi*. On the other hand, our results are in agreement with previous findings suggesting poor correlation between death and parasitemia level (Wrightsman et al. 1982, Minoprio et al. 1989). Precocious peak of parasitemia and high mortality obtained in Dm28c clone infected mice are different from those obtained in Balb/c mice infected with both chemically induced and triatomine-derived metacyclic trypomastigotes (Lopes et al. 1995). However these differences are not unexpected

since we have used both younger mice which are higher susceptible to *T. cruzi* infection and more infective trypomastigotes obtained from cell culture supernatant.

The low levels of parasitemia caused by *T. rangeli* and the briefness of its presence in experimental animals (Urdaneta-Morales & Tejero 1985) have made the study of this parasite difficult. In our hands, the parasitemia level in mice depends on the inoculum origin. Mice infected with parasites obtained from axenic culture showed very low parasitemia levels compared with mice infected with metacyclic trypomastigotes from haemolymph and salivary glands of *R. prolixus*. Differences with inocula of different origins using C23 strain of *T. rangeli* has been previously reported (Zúñiga et al. 1997b).

Despite its non-pathogenic characteristics for vertebrate hosts, *T. rangeli* infection induces a humoral immune response resulting in high levels of cross-reactive antibodies with *T. cruzi*, due to the similarity of their surface antigens (Grisard et al. 1999). On the other hand, mice immunized with fixed *T. rangeli* epimastigotes had significantly lower parasitemias and longer survival than controls when infected with virulent *T. cruzi* trypomastigotes (Basso et al. 1991, Introini et al. 1998).

When *T. cruzi* strains were challenged here into mice preinfected with *T. rangeli*, mortality decreased and parasitemia was more controlled suggesting that *T. rangeli* is able to induce a strong specific immune response controlling parasitic multiplication in infected individual (Avila & Rojas 1990, Introini et al. 1998). These results indicate that Choachí-2V strain is a very good antigen for the study of acquired immunity in Chagas disease. In countries where both parasites coexist, the natural exposure to non-virulent *Trypanosoma* strains may be involved in the diversity of clinical, pathological and immunological manifestations which occur following infection with the virulent *T. cruzi* strains.

The presence of *T. rangeli* in the same distribution area as *T. cruzi* in almost all the countries of South and Central America, has been largely considered a serious complication for the Chagas disease diagnosis (Grisard et al. 1999), since false-positive results can be expected from the cross-reactivitiy between both parasites. However, the results presented here suggest that occurrence of single and/or mixed infection in vertebrate hosts may have beneficial effects in evolution and epidemiology of Chagas disease. Protection against *T. cruzi* infection was induced by pre-infection with *T. rangeli* parasites. Peak parasitemia in pre-infected mice was significantly reduced and the animals were negative until at least 60 days p.i. The severity of tissue damage was clearly diminished and mice survived to infection. Therefore, the possibility that concomitant infection on endemic areas mask or naturally protect people from the development of severe symptoms of Chagas disease can not be excluded.

The use of different *T. cruzi* parasites, with distinct tissue tropism and virulence, may influence the type of effector response by selecting distinct T cell subsets with varying cytokines profiles (Rottenberg et al. 1988, Tarleton 1990, Tarleton et al. 1992). Therefore, although the immune mechanisms responsible for the resistance to *T. cruzi* infection are still unclear, innate and acquired humoral

and cellular immune response might be exacerbated in the *T. rangeli* pre-infected mice. We presumed that parasite-specific immunity is already operative in the 15 days of lag time difference between the *T. rangeli* and *T. cruzi* infections since *T. cruzi*-specific antibodies and delayed type hypersensitivity develop within 2 weeks during acute infection of A/J mice with the Brazil strain of the parasite (Leon & Egman 2001). During invasion of the host, the parasites expose functional molecules that become targets for the host immune response and may contribute to the killing of the parasite. Among these molecules, the several members of the Shed Acute Phase Antigen/Trans-sialidase/Neuraminidase (SAPA/TS/NS) protein family and the core protein of the mucin-like glycoprotein expressed in the bloodstream trypomastigotes are involved in both generating an immune response and helping to establish the parasite infection (Frasch 1994, 2000). The *trans*-sialidase, which is located in the surface membrane of *T. cruzi* and is also shed into the medium, enables the parasite to invade host cells by transferring sialyl residues from host glycoconjugates to the abundant mucin-like acceptor molecules on the parasite surface. Once sialylated, the acceptor glyconjugates seem to be involved in binding and invasion of the host cells by the parasite and in the protection against the alternative complement pathway (Ruiz et al. 1993, Schenkman et al. 1991, Tomlinson et al. 1994).

Antibodies recognizing the parasite mucins (Pollevick et al. 2000) and those neutralizing the *trans*-sialidase activity have been found in sera from *T. cruzi* infected humans and animals (Leguizamón et al. 1994a, b). Therefore, we can speculate that cross-reactive antibodies induced by the homologous mucins and sialidases expressed in *T. rangeli* might be involved in the protective effect of *T. rangeli* infection on evolution of Chagas disease.

The *T. cruzi* SAPA/TS/NS molecules are characterized by the presence of a carboxy-terminal domain made up of a variable number of tandemly repeated 12 aminoacid-long units (SAPA repeats) and a non-repeated amino-terminal domain containing the enzymatic activity. SAPA repeats are not required for *trans*-sialylation, but are necessary to maintain the enzyme active in circulation in the blood and to stimulate the production of antibodies against the catalytic domain that inhibits the *trans*-sialidase activity of the molecule (Buscaglia et al. 1998). *T. rangeli* expresses a sialidase, 70% identical in sequence to the *T. cruzi* enzyme but it lacks the SAPA repeats extension and is completely devoid of *trans*-sialylation activity (Buschiazzo et al. 1997). However, since a few aminoacid differences around the active site of *T. rangeli* sialidase and the *T. cruzi trans*-sialidase confer the transglicosylation activity (Buschiazzo et al. 2000), we can not exclude the possibility that antibodies against cross-reacting epitopes within the *T. rangeli* sialidase neutralize the enzymatic activity of the *T. cruzi trans*-sialidase.

In summary, the results presented here have demonstrated that Balb/c mice infected with *T. rangeli* are protected against lethal infection by virulent *T. cruzi* trypomastigotes. The minimal to discrete inflammatory tissue infiltrate in absence of parasites observed in protected animals is not unexpected since as suggested by others (Younés-Chennoufi et al. 1988, Guarner et al. 2001), the antigenic stimulation might persists throughout the chronic phase even though the parasites are not morphologically detectable.

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